The Catalytic Activity of Thermal Polyanhydro-α-amino Acids for the Hydrolysis of p-Nitrophenyl Acetate

(Catalysis by Thermal Polyamino Acids)

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ABSTRACT

Thermally prepared polyanhydro-α-amino acids containing the eighteen amino acids common to protein accelerate the hydrolysis of p-nitrophenyl acetate. Such action is catalytic, rather than stoichiometric. Histidine residues play a key role in the hydrolysis; the contribution to activity of residues of lysine and arginine is minor, and no activity is observed for polymers containing no basic amino acids. All polymers tested were more active than the equivalent amount of histidine, some being more than ten times as active. The higher levels of activity, per unit of histidine, are observed with those polymers that contain a relatively low proportion of histidine.

INTRODUCTION

The preparation of a variety of polyanhydro-α-amino acids by the simple process of heating the dry monomers has been documented (1 and bibliography). The thermal polymers thus prepared range in compositional complexity from homopolymers (2,3) to pan-polymers (4) that contain some proportion of each of the eighteen amino acids commonly found in protein. The latter thermal polymers exhibit many properties in common with protein (4,5); they have been termed proteinoids (4). These polymers can be regarded as easily varied models of protein (1,5,6).

The catalytic activity of thermal polyanhydro-α-amino acids has especially invited attention. The first substrate for which such activity was reported was the unnatural ester, p-nitrophenyl acetate (NPA; 5,7,8). Thermal proteinoids have been shown to accelerate the formation, and subsequent decarboxylation, of glucuronic acid from glucose (9), the decarboxylation of pyruvic acid (10,11), and the conversion of oxaloacetic acid to pyruvic acid (12). The zinc salts of acid proteinoid accelerate the hydrolysis of ATP (13), as earlier observed with inorganic salts of zinc (14) at a higher temperature.

The present paper reports additional details on the behavior of proteinoids in the hydrolysis of NPA. Polymers that were designed to contain varying proportions of histidine have been employed. Attention to histidine residues was influenced by the indications that peptide-linked histidine is involved in the active center of several hydrolytic enzymes (15-22), and by the fact that various compounds containing the imidazole ring have

been found to be catalytically active in enzyme-model experiments (23-36), which frequently have used NPA as the substrate. Histidine residues have been found to play a key role in the action of proteinoids on NPA. Data supporting the inference that the action is catalytic have been obtained.

MATERIALS AND METHODS

Proteinoids were prepared from reactants consisting of 2 parts by weight each of aspartic and glutamic acids, 1 part of an equimolar mixture of the 16 common neutral and basic amino acids (4), with varying proportions of histidine hydrochloride hydrate. The reactants were heated in an oil bath at 170°C, under nitrogen, for 3 hrs. The crude products were dissolved in either 5% or 10% sodium bicarbonate, were dialyzed against distilled water for 3-5 days, and the resulting solutions were dried. Yields of soluble, non-diffusible material were generally 2-4 weight %.

L-Histidine was obtained from Nutritional Biochemicals Corporation and from California Biochemical Corporation, various lots being used. p-Nitrophenol was an Eastman product. p-Nitrophenyl acetate (NPA) was prepared by the method of Chattaway (37), uncorr. m.p. 76-78°C [lit., 83°C (37); 79.5-80°C (38)]. Gratitude is expressed to Dr. H. C. White of the Dow Chemical Company for generous gifts of various amino acids.

The contents of histidine in the intact polymers were determined by the method of Macpherson (39), with polymers lacking histidine used as blank controls, and histidine as a standard (cf. 32). Values obtained on the intact polymers were in close

agreement with those obtained on acid hydrolysates (7).

Solutions for catalytic assay were 2×10^{-3} M in NPA, 2% in acetone, and contained 4.0 mg. of proteinoid in 0.067 M phosphate buffer, pH 6.2, in a total volume of 10.0 ml., 31-32°C (cf. 23). The liberated p-nitrophenol was assayed at 400 mμ with a Bausch and Lomb Spectronic 20 colorimeter or with a Beckman DU spectrophotometer. Initial rates were calculated from the slopes of progress curves (which were linear for ca. 1 hr., with less than 10% of the NPA being hydrolysed) and were related to the rate observed for the "spontaneous" hydrolysis control, assigning to the latter a value of 1.00. The activities of the polymers, after correcting for the spontaneous rate, were related to that of free histidine by dividing by the value for the equivalent amount of histidine obtained either by simultaneous comparison or from a linear plot of activity versus histidine conon.

Acetate produced from the polymer-catalyzed hydrolysis of NPA was demonstrated by titrating at constant pH 6.2 with 0.0146 N KOH on a Radiometer Titrigraph.

RESULTS AND DISCUSSION

The histidine contents of proteinoids (Table I) were directly proportional to the amount of this amino acid present in the reactants. The mean ratio of histidine in the product (26 polymers) to histidine in the reactants was 0.38, std. dev. ±0.045, or ±12 relative %. The small std. dev. indicates that the incorporation into proteinoids of this amino acid, like others (6) is highly controllable and reproducible.

TABLE I

Histidine Content and Catalytic Activity for Hydrolysis of HPA of Representative Proteinoids

Proteinoid[a]	Histidine Content, wt. %[b]	Catalytic Activity[c] Relative to Spontaneous Hydrolysis, and Std. Dev.	Relative to Equivalent Amount of Histidine
A-1.3	0.6	1.29 ± 0.06	3.6
A-2.8	0.9	2.64 ± 0.19	14
A-2.8, hydrolyzate	-	1.28 -	-
A-4.8	1.8	1.42 ± 0.09	1.8
A-8.0	3.2	2.02 ± 0.11	2.4
A-13.5	-	2.18 ± 0.17	-
B-2.3	0.9	1.61 ± 0.34	5.1
B-13.5	4.6	1.91 ± 0.05	1.5
D-1.3	0.5	1.24 ± 0.04	3.4
D-2.8-a	1.0	1.54 ± 0.12	4.2
D-2.8-b	1.2	1.21 ± 0.01	1.3
D-2.8-d	1.1	1.30 ± 0.01	2.1
D-4.8	1.9	1.41 ± 0.05	1.6
D-8.0	2.6	1.65 ± 0.09	1.9
D-13.5	4.0	1.95 ± 0.12	1.8
E-1.3	0.4	1.61 ± 0.24	12
E-6.7	3.0	1.52 ± 0.21	1.3
E-8.0	3.0	1.65 ± 0.31	1.6

[a] Identical capital letters in the polymer code indicate that the polymers were prepared at the same time. The numerical values refer to the weight % histidine, present as the HCl hydrate, but expressed as the free base, present in the reaction mixtures. Lower case letters following identical numbers designate replicate samples prepared simultaneously from reactants of identical composition.

[b] Evaluated on the intact polymers by the method of Macpherson (39) and expressed as the free base.

[c] The components of the assay solutions are described in the METHODS section; polymer concn. was 0.4 mg/ml.

[d] Obtained as described in the METHODS section.

The catalytic activities on NPA of several representative proteinoids are recorded in Table I. A total of 26 histidine-containing proteinoids were evaluated. The degree of activity per unit weight of polymer, relative to the spontaneous control, tends to increase as the proportion of histidine in the polymer increases. Exceptions, however, are observed (e.g., polymers A-2.8, D-2.8-a, and E-1.3). When activity is expressed relative to that of the equivalent amount of free histidine, several polymers with a relatively low content of histidine are more active. The mean level of activity for 11 polymers containing from 1.8 - 4.6% histidine was only 1.7 (std. dev. ± 0.28), whereas 13 polymers containing from 0.4 to 1.2% histidine have an average value of activity of 4.6 (std. dev. ± 3.9); two of the latter polymers were more than 10 times as active as the equivalent amount of histidine. The amino acids contained in the hydrolysate of A-2.8 are much less active than is the parent polymer. Comparison of the activities on NPA of proteinoids with those of a large number of other histidine-containing compounds (cf., for example, 23-25,29,30,32,40), including peptides and polyamino acids, indicates that several of the thermal polymers are among the more active synthetic derivatives of histidine yet to be reported.

A series of polymers was prepared from which basic amino acids were systematically omitted. No activity was observed with the polymer lacking all 3 common basic amino acids, those polymers containing lysine and/or arginine, but not histidine, exhibited a low level of activity (average value, relative to the

spontaneous control, 1.05), whereas polymers containing histidine had an appreciably higher level of activity (average value, 1.20). The presence of lysine and/or arginine in the polymers with histidine did not appreciably alter the level of activity. It would thus appear that any contribution to activity by lysine or arginine residues is minor. These results are consistent with the finding that, on a molar basis, free lysine and free arginine at pH 6.8 were only 0.16 and 0.06 times as active, respectively, as free histidine. A low level of activity for lysine in homopolymeric form has been reported (32).

Titrimetric evaluation of the hydrolysis of NPA at constant pH 6.2 revealed that the liberation of p-nitrophenol was accompanied by the release of an equivalent amount of acetate. For example, in duplicate analyses (5 μmoles NPA and 6 mg. polymer in 5 ml. buffer, 4 hr., 30°C), 1.22 and 1.14 μmoles of p-nitrophenol were detected colorimetrically and, after correcting for the degrees of dissociation of p-nitrophenol (38) and acetate, 1.21 and 1.15 μmoles of acetate were found titrimetrically. In the same time period, the spontaneous control liberated less than half these amounts of products. Such results have been noted both with proteinoids and with thermal copolymers containing only aspartic acid and histidine.[a]

[a]The use in similar experiments of polymers containing residues of aspartic acid which were not previously converted (41) from the cyclic imide (2) to the peptide form resulted in some uptake of base in the absence of NPA, due to the hydrolysis of imide. However, after correcting for such uptake, liberation of acetate from NPA was evident.

Several kinds of evidence indicate that the reactions are catalytic, rather than stoichiometric. When the hydrolysis of 10^{-3} M NPA by proteinoid A-2.8 (0.2 mg./ml.) was followed to completion, linear first order plots were obtained for at least 90% of the reaction. At the ratio of substrate to polymer employed (ca. 75 to 1, expressing the molar concn. of polymer in terms of histidine residues), non-linear first order plots would be predicted for a stoichiometric reaction (28,41). After all of the NPA had reacted, a second aliquot of NPA was added to the solution, giving 10^{-3} M NPA. The first order rate constant noted with the second aliquot of NPA was essentially identical to those observed with the first. A lower rate would be predicted for the second aliquot if the reaction were stoichiometric (31). (The first order rate constant for the proteinoid, corrected for the spontaneous control and converted to unit concn. of 1 mg./ml., was 14×10^{-2} per hour; a value of 4×10^{-2} per hour was obtained for the spontaneous control.) The molar ratio of NPA hydrolyzed per histidine residue, after correcting for the NPA hydrolyzed spontaneously, is calculated from the results observed with both aliquots of NPA to be at least 25:1, a result further indicating catalysis. The liberation of acetate and p-nitrophenol in equal proportions is also consistent with an inference of catalysis. The action of thermal polyanhydro-α-amino acids on oxaloacetic acid has also been shown to be catalytic (12). [Although aseptic precautions were not taken in the prolonged (several days) experiment mentioned above, no growth of microorganisms was observed when aliquots of the assay solutions were plated on nutrient

agar. This observation, which is in accord with the known toxic nature of p-nitrophenol (42), indicates that the results are not due to contaminating microbes.]

The truly catalytic action of thermal polyanhydro-α-amino acids on NPA, the relatively high level of activity shown by some preparations, and the ease of variation of the amino acid composition of the thermal polymers (resulting in different levels of activity) suggest the use of the thermal polymers as an alternate type of model compound for the investigation into the kinds of amino acids involved in the active sites of enzymes. An additional feature of relevance in this context is that thermal polymers (both proteinoids and copolymers of aspartic acid and histidine) are inactivated by heating their buffered solution, a topic that is treated in a subsequent report (43). Also, because proteinoids are prepared under geological conditions (1), the present results suggest one way in which macromolecular catalysts could have been formed abiotically from amino acids, thence to become attributes of the primordial cell (1).

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